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Polyamine depletion arrests cell cycle and induces inhibitors p21^{Waf1/Cip1}, p27^{Kip1}, and p53 in IEC-6 cells

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Ray, Ramesh M., Barbara J. Zimmerman, Shirley A. McCormack, Tarun B. Patel, and Leonard R. Johnson. Polyamine depletion arrests cell cycle and induces inhibitors p21^{Waf1/Cip1}, p27^{Kip1}, and p53 in IEC-6 cells. *Am. J. Physiol. Cell Physiol.* 45: C684–C691, 1999.—The polyamines spermidine and spermine and their precursor putrescine are intimately involved in and are required for cell growth and proliferation. This study examines the mechanism by which polyamines modulate cell growth, cell cycle progression, and signal transduction cascades. IEC-6 cells were grown in the presence or absence of DL- α -difluoromethylornithine (DFMO), a specific inhibitor of ornithine decarboxylase, which is the first rate-limiting enzyme for polyamine synthesis. Depletion of polyamines inhibited growth and arrested cells in the G₁ phase of the cell cycle. Cell cycle arrest was accompanied by an increase in the level of p53 protein and other cell cycle inhibitors, including p21^{Waf1/Cip1} and p27^{Kip1}. Induction of cell cycle inhibitors and p53 did not induce apoptosis in IEC-6 cells, unlike many other cell lines. Although polyamine depletion decreased the expression of extracellular signal-regulated kinase (ERK)-2 protein, a sustained increase in ERK-2 isoform activity was observed. The ERK-1 protein level did not change, but ERK-1 activity was increased in polyamine-depleted cells. In addition, polyamine depletion induced the stress-activated protein kinase/c-Jun NH₂-terminal kinase (JNK) type of mitogen-activated protein kinase (MAPK). Activation of JNK-1 was the earliest event; within 5 h after DFMO treatment, JNK activity was increased by 150%. The above results indicate that polyamine depletion causes cell cycle arrest and upregulates cell cycle inhibitors and suggest that MAPK and JNK may be involved in the regulation of the activity of these molecules.

ornithine decarboxylase; DL- α -difluoromethylornithine; putrescine; signal transduction; mitogen-activated protein kinase; cyclin-dependent kinase inhibitor

THE POLYAMINES SPERMIDINE and spermine and their precursor putrescine are intimately involved in and required for cell growth and proliferation (43, 57). Intracellular polyamine levels are highly regulated and are primarily dependent on the activity of ornithine decarboxylase (ODC), which catalyzes the first rate-limiting step in polyamine biosynthesis (48, 37). An increase in ODC activity is one of the earliest biochemical events associated with the induction of cellular proliferation (43, 57), and depletion of polyamines by DL- α -difluoromethylornithine (DFMO), a specific and irreversible inhibitor of ODC, attenuates trophic re-

sponses in a number of tissues (43, 37). Although the importance of polyamines in cell growth is well known, their exact role in specific events related to cell proliferation at the molecular level is still unclear.

Growth regulatory consequences of polyamine depletion suggest a link between signal transduction cascades, cell cycle machinery, and apoptosis. Several investigators have provided evidence for polyamine-dependent restriction points during the G₀-G₁ transition and G₁ phase in various cell types (5, 18, 51). So far, the precise location of the block has not been determined. Orderly progression through the cell cycle is now known to be dependent on the coordinated interaction between key cell cycle regulatory molecules including cyclins, cyclin-dependent kinases (CDKs), and CDK inhibitory proteins such as p21^{Waf1/Cip1} and p27^{Kip1} (20, 22, 25, 42). Increased levels of CDK inhibitory proteins prevent the release of E2F, a transcription factor required for the transcription of S phase genes, from retinoblastoma protein, resulting in cell cycle arrest (6, 7, 11, 33).

It is now established that the mitogen-activated protein kinase (MAPK) cascade is also involved in a variety of biological processes, such as differentiation (9, 58), cell attachment (8), cell motility (26), smooth muscle contraction (1), and protein synthesis (53), depending on the stimuli and cell type. For example, in PC-12 cells, epidermal growth factor (EGF) activation of the MAPK pathway leads to proliferation, whereas nerve growth factor-stimulated MAPK activation leads to differentiation (9, 58). Recently, a novel paradigm has been described in which the MAPK kinase (MEK)/MAPK pathway negatively regulates CDK activity and mediates cell cycle arrest (45).

The c-Jun NH₂-terminal kinase [JNK; also known as stress-activated protein kinase (SAPK)] group of MAPKs is activated by exposure of cells to environmental stress or by treatment of cells with proinflammatory cytokines (17, 21, 28, 56), ultraviolet light (10), DNA-damaging drugs (59, 64), protein synthesis inhibitors (21), ceramide (62), and tumor necrosis factor- α and interleukin-1 (28, 47). In addition, JNK activity is also induced by mitogenic signals, including growth factors (40) and oncogenic Ras (10). One potential function of JNK may be the initiation of programmed cell death (apoptosis). Overexpression of MEK kinase, the JNK kinase kinase, has a lethal effect on fibroblasts (23, 30). In addition, the tumor suppressor p53, which is essential for radiation-induced apoptosis (40), may be a substrate of JNK-1 *in vivo* (39).

In the small intestine, cell proliferation and cell migration are fundamental processes for the organiza-

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tion and maintenance of tissue integrity. Highly regulated mechanisms appear to control the rate of cell proliferation, migration, and differentiation of the crypt cells. Frey et al. (15) established that increases in p21^{Waf1/Cip1} and p27^{Kip1} and hypophosphorylation of retinoblastoma protein are associated with phorbol ester-mediated activation of the protein kinase C isoform and cell cycle arrest in IEC-18 cells. Sustained elevation of JNK and p53 has been reported to induce apoptosis in several cell lines (8, 32). Spontaneous apoptosis is part of the stem cell homeostatic process, ensuring that extra stem cells are deleted from the small intestine. Addition of a single stem cell to the normal complement of 250 cells in a small intestinal crypt could distort the architecture of the crypt and generate a hyperplastic crypt, which is rare in the small intestine, implying that stem cell number is tightly regulated. Our laboratory has been interested in the role of polyamines in the growth and repair of the gastrointestinal mucosa and has examined these processes in both rats and in cultured, normal rat intestinal epithelial cells (IEC-6). Recently, we demonstrated that, in IEC-6 cells, EGF treatment decreased the doubling time by 8 h, whereas, in polyamine-depleted cells, EGF had no effect on the doubling time. An inhibitory effect of polyamine depletion on phosphorylation of the EGF receptor suggested the involvement of polyamines in signaling cascades (35). However, the precise role of polyamines in the signaling cascade that includes MAPK, JNK, and cell cycle regulators has not been investigated. In this report, data are presented that show the induction of p53 and CDK inhibitory proteins upon polyamine depletion and cell cycle arrest. Concomitant induction of MAPK and JNK during polyamine depletion suggests a link between the signal transduction cascade and negative growth regulation in IEC-6 cells.

MATERIALS AND METHODS

Materials. Medium and other cell culture reagents were obtained from GIBCO BRL (Grand Island, NY). Fetal bovine serum (FBS), dialyzed fetal bovine serum (dFBS; 1,000-molecular weight cutoff), myelin basic protein (MBP), and propidium iodide were from Sigma (St. Louis, MO). [γ -³²P]ATP and an enhanced chemiluminescence Western blot detection system were purchased from DuPont-NEN (Boston, MA). DFMO was a gift from the Merrell Dow Research Institute of Marion Merrell Dow (Cincinnati, OH). Both anti-MAPK extracellular signal-regulated kinase (ERK)-2 (monoclonal) and anti-MAPK ERK-1-CT (polyclonal) antibodies were purchased from Upstate Biotechnology (Lake Placid, NY). JNK-1, p21, p27, and p53 antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). The apoptosis detection kit was purchased from Oncor (Gaithersburg, MD). TACS apoptotic DNA laddering kit was purchased from Trevigen (Gaithersburg, MD). The IEC-6 cell line (American Type Culture Collection CRL-1592) was obtained from the American Type Culture Collection (Manassas, VA) at passage 13. The cell line was derived from normal rat intestine and was developed and characterized by Quaroni et al. (46). The cells are nontumorigenic and retain the undifferentiated character of epithelial stem cells. All other chemicals were of the highest purity commercially available.

Cell culture. The IEC-6 cell stock was maintained in T-150 flasks in a humidified, 37°C incubator in an atmosphere of 90:10 air-CO₂. The medium consisted of DMEM with 5% heat-inactivated FBS and 10 µg/ml insulin and 50 µg/ml gentamicin sulfate. The stock was passaged weekly at 1:10 and fed three times per week, and passages 15–20 were used. For the experiments, the cells were taken up with 0.05% trypsin plus 0.53 mM EDTA in Hanks' balanced salt solution without calcium and magnesium and counted by hemocytometer.

Growth studies. The cells were plated at a density of 6.25×10^4 cells/cm² in T-25 flasks in DMEM containing 5% dFBS plus 10 µg/ml insulin and 50 µg/ml gentamicin sulfate (DMEM-dFBS) with or without DFMO or putrescine. Each experiment contained three each of untreated, DFMO-treated, and DFMO/putrescine-treated flasks. Every other day, the cells in three flasks from each group were taken up with trypsin-EDTA and the number of cells in an aliquot was counted by Coulter counter. Each experiment was carried out in triplicate, the controls were combined, and the data were expressed as percentages of control.

Apoptosis. Cells were plated as described above in DMEM-dFBS with or without DFMO or putrescine on chambered slides. On day 5, the cells were fixed in 4% neutral buffered Formalin and postfixed in 2:1 ethanol-acetic acid. Apoptotic cells were identified by terminal deoxynucleotidyl transferase labeling according to the manufacturer's instructions. Following the procedure, the cellular DNA was counterstained with propidium iodide (5 µg/ml). The nucleosomal fragmentation assay was carried out by isolating DNA from the cells using the TACS apoptotic DNA laddering kit and analyzing the DNA by agarose gel electrophoresis following the manufacturer's instructions.

Cell cycle analysis. Cells were plated in DMEM-dFBS with or without DFMO in T-25 flasks at a density of 6×10^4 cells/cm². They were taken up with trypsin-EDTA on days 1–4 postplating. The cells were collected by centrifugation for 5 min at 100 g, washed with ice-cold PBS-1% BSA (washing buffer), resuspended in 0.5 ml, and fixed with 1 ml 70% ethanol at –20°C, added dropwise. The cells were washed three times in washing buffer at 4°C and resuspended in 1 ml containing 100 µg RNase (RASE, Worthington) and 5 µg propidium iodide. They were then incubated at 37°C for 15 min in the dark, washed three times with 3 ml of washing buffer, resuspended in 1 ml, and analyzed by flow cytometry.

Preparation of cell extract. The IEC-6 cells were plated (day 0) in 60-mm dishes at a density of 6.25×10^4 cells/cm² in DMEM-dFBS with or without DFMO (5 mM) and putrescine (10 µM). Dosages were chosen from dose-response curves carried out previously. On day 3, the medium was removed and medium was added that did not contain dFBS but did contain DFMO or DFMO plus putrescine when required. On day 4, medium was removed and 500 µl of cold immunoprecipitation buffer (IPB; 10 mM Tris-HCl at pH 7.4, 1% Triton X-100, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 0.5% Nonidet P-40, 1 mM phenylmethylsulfonyl fluoride, 10 mM NaF, 200 µM Na₃VO₄, 80 µg/ml leupeptin, and 40 µg/ml aprotinin) was added. The cell suspension was rotated at 4°C for 30 min, and the extract was cleared by centrifugation at 10,000 g for 5 min. The extracts were stored at –80°C until use. Protein concentration was determined by the method of Bradford (4), using BSA as a standard.

Immunoprecipitation. Cell lysates were matched for protein and precleared with 20 µl of protein A/G agarose for 1 h at 4°C. The precleared supernatants were further incubated overnight with 2 µg of antibody recognizing p21, p27, p53, ERK-1, ERK-2, or JNK-1, and immunocomplexes were pread-

sorbed to protein A/G agarose. Preadsorbed immunocomplexes were then used to measure ERK-1, ERK-2, or JNK activity by *in vitro* kinase assay and p21, p27, and p53 by Western blotting.

In vitro MAPK assay. The immunocomplexes were pelleted by centrifugation (10,000 *g* for 2 min) and then washed three times with IPB and once with kinase assay buffer [KAB; 20 mM Tris-HCl, pH 7.4, 10 mM MgCl₂·6H₂O, 200 μM Na₃VO₄, 10 mM NaF, and 1 mM dithiothreitol (DTT)]. The pellet was resuspended in 40 μl KAB containing 0.3 mg/ml MBP, 20 μM ATP, and 10 μCi [γ -³²P]ATP. After 20 min at 30°C, the reaction was terminated by the addition of 40 μl of 2× Laemmli sample buffer. Samples were then boiled for 5 min and subjected to 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed for 1–3 h to Kodak X-ray film.

In vitro JNK assay. The immunocomplexes were pelleted by centrifugation (10,000 *g* for 2 min) and then washed three times with JNK buffer and once with JNK assay buffer. The agarose beads were resuspended in 30 μl JNK assay buffer (20 mM HEPES, pH 7.6, 20 mM MgCl₂, 20 mM β-glycerophosphate, 2 mM DTT, 100 μM *p*-nitrophenyl phosphate, 100 μM Na₃VO₄), 1 μg of glutathione *S*-transferase (GST)-c-Jun protein, 20 μM ATP, and 10 μM [γ -³²P]ATP. After 20 min at 30°C, the reaction was terminated by the addition of 30 μl of 2× Laemmli sample buffer. Samples were then boiled for 5 min and subjected to 15% SDS-PAGE. The gels were stained with Coomassie brilliant blue, dried, and exposed for 1–3 h to Kodak X-ray film.

Western blot analysis. Cell extracts were prepared as described above. Total cell protein (50 μg) was separated on 15% SDS-PAGE gels and transferred to nitrocellulose membranes for Western blotting. Equal loading of protein was confirmed by staining the nitrocellulose membrane with Ponceau S. The membranes were then probed with an antibody directed against one of the proteins (p21, p27, p53, ERK-1, ERK-2, and JNK-1). The immunocomplexes were visualized by the enhanced chemiluminescence detection system and quantitated by densitometric scanning.

RESULTS

Polyamine depletion inhibits growth but does not induce apoptosis in IEC-6 cells. Several investigators have demonstrated that polyamines are required for cell growth and proliferation (43, 48, 57). In this and other studies (35), we have shown that depletion of cellular polyamines by long-term administration of DFMO significantly decreases IEC-6 cell growth. Decreased cell growth occurred within 3–4 days following DFMO treatment and remained unchanged (Fig. 1). Supplying exogenous spermidine prevented the decrease in rate of growth. Similar restoration of growth occurs with putrescine or spermine. Recently, studies have shown that increases in JNK result in the induction of apoptosis in certain cell types (8). We wanted to examine whether the reduction in growth in polyamine-depleted cells was a result of apoptosis. By examining the terminal deoxynucleotide transferase labeling of 3'-OH ends of DNA (Fig. 2A) and DNA laddering (Fig. 2B) as markers of apoptosis, we found that treatment of IEC-6 cells with DFMO did not result in the induction of cell death.

Polyamine depletion affects the progression of the IEC-6 cell cycle. The increased doubling time and

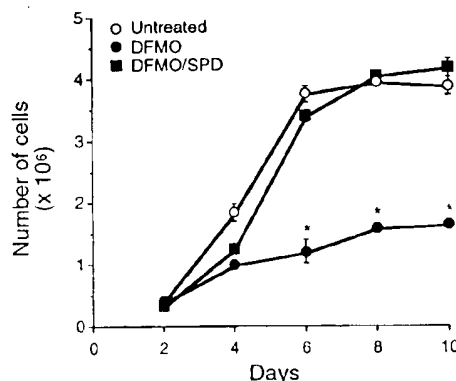


Fig. 1. Polyamine depletion decreases cell growth. Growth curves of IEC-6 cells that were grown under control conditions or in presence of 5 mM DL- α -difluoromethylornithine (DFMO) or DFMO + 5 μM spermidine (SPD) (means \pm SE, *n* = 3). *Significant difference compared with untreated controls using ANOVA (*P* < 0.05).

decreased cell number without apoptosis suggest that polyamines might be involved in the regulation of the cell cycle. Therefore, we examined the effect of DFMO on the progression of the cell cycle. In controls, normal cell cycle progression was evident by an increased S phase with a concomitant decrease in the G₁ phase on days 1 and 2 and then subsequent accumulation of cells into G₁ on day 3 when cells reached confluence (Fig. 3A). In contrast to this, polyamine depletion arrested cells in the G₁ phase on day 1, as shown in Fig. 3B. About 60% of the cells were in the G₁ phase, and this increased on subsequent days. Addition of putrescine to DFMO-treated cultures caused the normal progression of the cell cycle to resume (data not shown).

Polyamine depletion induces p53, p21^{Waf1/Cip1}, and p27^{Kip1}. As shown in Fig. 4, DFMO treatment of IEC-6 cells results in the accumulation of proteins that inhibit cell cycle progression. The level of p53, a tumor suppressor gene product, was increased by 150% in DFMO-treated cells (Fig. 4A); p27^{Kip1}, a mitotic inhibitor protein, was upregulated in polyamine-depleted cells by 50% (Fig. 4B). Polyamine depletion also resulted in a remarkable increase (77%) in the level of p21^{Waf1/Cip1} (Fig. 4C), which negatively regulates the formation of cyclin-CDK complexes that are required for normal cell cycle progression. These results support the notion that polyamines normally suppress pathways that increase CDK inhibitory protein expression.

Effect of polyamine depletion on the activities and levels of ERKs. We previously reported that polyamines are required for EGF-mediated cell proliferation in IEC-6 cells (35). It is now well established that the EGF-mediated signal cascade that involves ERKs (ERK-1, ERK-2, and JNK, also called SAPK) plays important roles in cell proliferation (50). Phosphorylation of transcription factors such as c-Fos, c-Myc, and c-Jun by activated ERKs increases the rate of transcription of growth-related genes. Previous studies from this laboratory have established that DFMO treatment depleted putrescine within 3 h, but depletion of spermidine required 24 h and depletion of spermine required

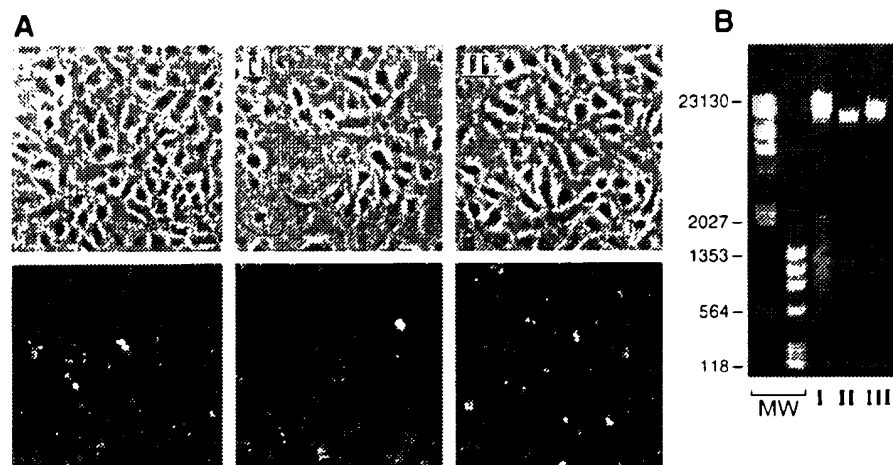


Fig. 2 Polyamine depletion does not induce apoptosis in IEC-6 cells. *A*: cells were grown for 4 days under control conditions (*I*) or in presence of DFMO (*II*) or DFMO + putrescine (*III*) and then were subjected to terminal deoxynucleotidyl transferase labeling. Immunofluorescence analysis of each slide is shown below corresponding phase-contrast photomicrograph. *B*: cells were grown as described above, and isolated DNA was subjected to agarose gel electrophoresis to determine nucleosomal cleavage by DNA laddering assay. Molecular weight (MW) lanes are DNA size markers λ Hind III digest (*left*) and ϕ X174 Hae III digest (*right*).

3 days (36). We examined the activities of ERK-1 and ERK-2 by phosphorylation of MBP and JNK activity by phosphorylation of GST-c-Jun protein in DFMO-treated cells (3 days), which were then serum deprived to achieve quiescence. Polyamine depletion caused an ~50% increase in ERK-1 activity, and supplementation with putrescine restored the basal level of ERK-1

activity (Fig. 5, *A* and *B*). Polyamine depletion had only a marginal effect on the level of ERK-1 protein (Fig. 5*C*). In contrast, polyamine depletion increased ERK-2 activity 150% compared with control (Fig. 6). The increase in ERK-2 activity was largely prevented by addition of putrescine to DFMO-treated cells (Fig. 6, *A* and *B*). Interestingly, polyamine depletion significantly reduced the level of ERK-2 protein, which was also prevented by putrescine (Fig. 6*C*). Similarly, the activity of JNK was elevated upon polyamine depletion (120%) and remained elevated in the presence of putrescine (Fig. 7, *A* and *B*). The level of JNK protein was low in polyamine-depleted cells and returned to normal with the addition of putrescine (Fig. 7*C*). The induction of JNK activity was the earliest response to polyamine depletion, increasing within 5 h of DFMO treatment (Fig. 8). This increase was prevented by putrescine.

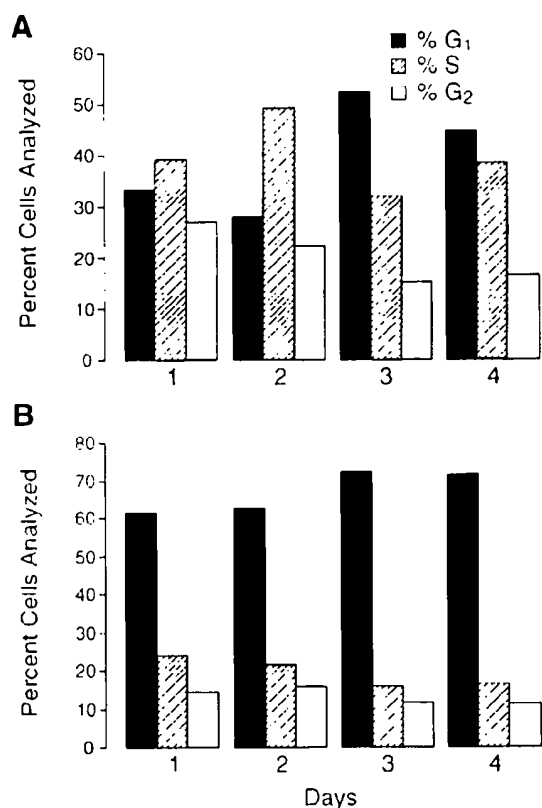


Fig. 3 Effect of polyamine depletion on cell cycle progression. IEC-6 cells were grown in medium without (*A*) and with (*B*) DFMO. At indicated time intervals, cells were processed as described in MATERIALS AND METHODS and subjected to flow cytometric analysis. Percent ages of total cells analyzed that are in G₁, S, and G₂ phases are shown from a representative of 3 experiments.

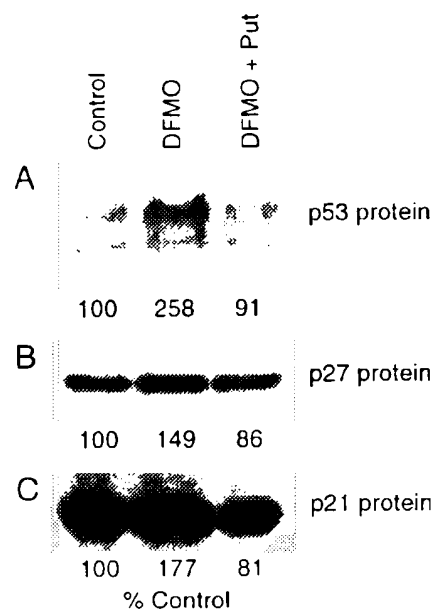


Fig. 4. Polyamine depletion increases levels of p53 (*A*), p27 (*B*), and p21 (*C*) proteins in IEC-6 cells as indicated by Western blot analysis.

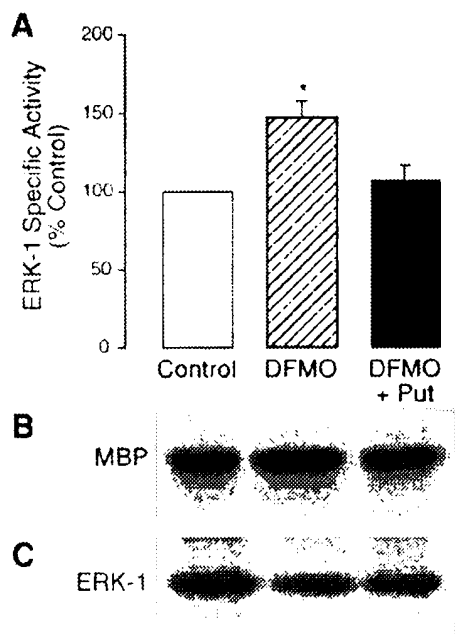


Fig. 5. Effect of polyamine depletion on activity and level of extracellular signal-regulated kinase (ERK) 1. IEC-6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, ERK 1 activity was measured by phosphorylation of myelin basic protein (MBP, *B*) and protein level (*C*) was analyzed by Western blot analysis. Specific activity (*A*) is expressed as %control (means \pm SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$).

These data indicate that polyamines influence the activities and protein levels of signal-regulated proteins in IEC-6 cells. These observations suggest that increases in the activities of ERKs as a result of polyamine depletion may be related to the inhibition of cell proliferation.

DISCUSSION

The rapidly proliferating cells of the mucosal epithelium of the small and large intestines constitute a dynamic and unique system to study factors regulating cell growth and differentiation. Increased understanding of the molecular factors that promote growth and maturation of the intestinal epithelium has considerable therapeutic relevance. The gastrointestinal mucosa has the ability to repair itself rapidly after damage. Repair of the damaged mucosa, called mucosal restitution or reepithelialization, consists of the sloughing of damaged epithelial cells, movement of surrounding cells to cover the wounded area (migration), and finally replacement of lost cells by the process of cell division (proliferation) (55). A series of studies from this laboratory established that polyamines are essential for the normal repair of gastric and duodenal erosions induced in a rat stress ulcer model. DFMO, an enzyme-activated inhibitor of ODC, almost completely prevents healing, and oral administration of polyamines immedi-

ately after the period of stress restores the normal rate of healing (60, 61). Although it has been repeatedly demonstrated that, in most cells, polyamine depletion leads to an inhibition of cell proliferation, the actual mechanism by which this is achieved is not understood at the molecular level. In view of the above, in the present investigation we demonstrate that polyamine depletion leads to cell cycle arrest with the induction of p53, p27, and p21 and involvement of sustained induction of the MAPK signal transduction cascade.

Because polyamine depletion had such a dramatic effect on cell growth (Fig. 1), we presumed that polyamines might have some regulatory role in cell cycle progression. IEC-6 cell cycle analysis was carried out in the presence and absence of DFMO to establish the specific effect of polyamine depletion on cell growth. Results in Fig. 3*B* clearly indicate that polyamine depletion arrested cells in the G_1 phase within 24 h (i.e., within one cell cycle). Seidenfeld et al. (51) also showed a marked increase in the G_1 phase fraction and decrease in the S phase fraction as a consequence of DFMO treatment in four different carcinoma cell lines. More recently, Fredlund and Oredsson (13, 14) found impaired DNA replication and lengthening of the G_1

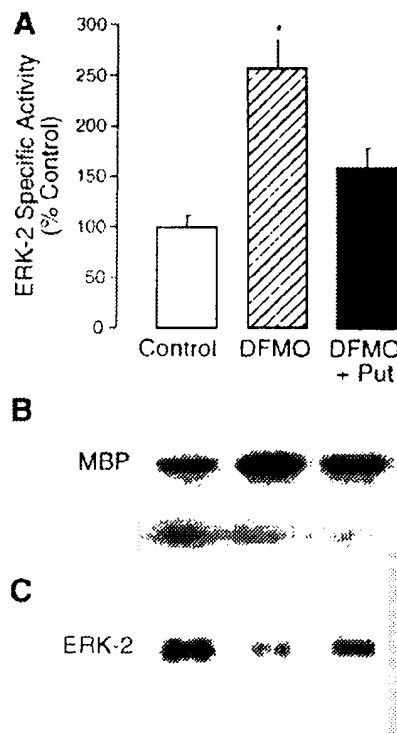


Fig. 6. Effect of polyamine depletion on activity and level of ERK-2. IEC-6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, ERK-2 activity was measured by phosphorylation of MBP (*B*) and protein level (*C*) was analyzed by Western blot analysis. Specific activity (*A*) is expressed as %control (means \pm SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$).

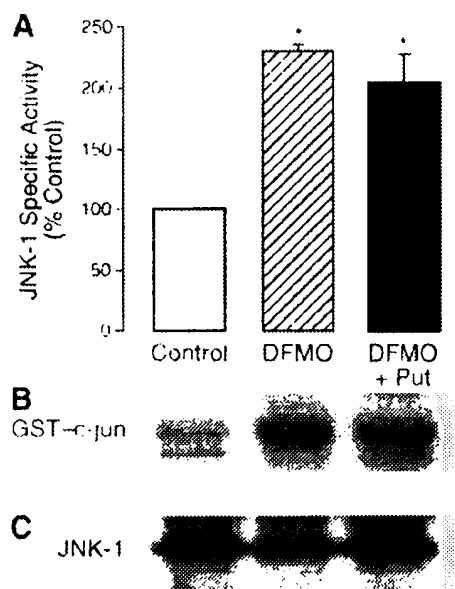


Fig. 7. Effect of polyamine depletion on activity and level of c-Jun NH₂-terminal kinase (JNK) 1. IEC 6 cells were grown under control conditions or in presence of DFMO or DFMO + putrescine for 3 days and serum starved for 24 h, and then cell extracts were prepared. After immunoprecipitation, JNK 1 activity was measured by phosphorylation of glutathione S transferase (GST) c-Jun (B) and protein level (C) was analyzed by Western blot analysis. Specific activity (A) is expressed as %control (means \pm SD, $n = 3$) and was calculated from arbitrary units of activity and protein levels determined by densitometric scanning. *Significant difference compared with control using ANOVA ($P < 0.05$).

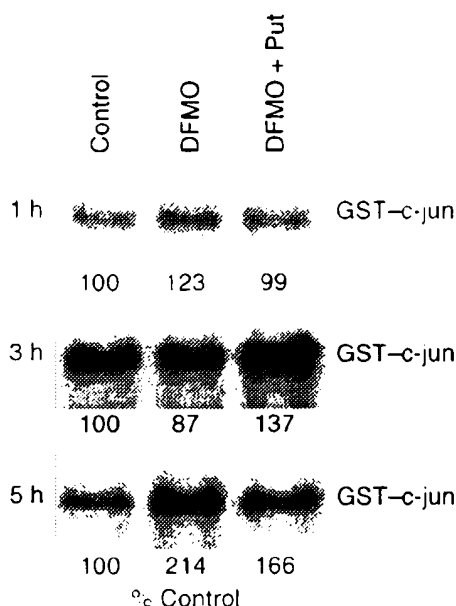


Fig. 8. Time course of effect of polyamine depletion on activity of JNK 1. IEC 6 cells were grown under control conditions for 3 days, serum starved for 24 h, and treated in presence and absence of DFMO or DFMO + putrescine for indicated times. JNK-1 activity was determined as described in MATERIALS AND METHODS.

and S phases within one cell cycle after plating Chinese hamster ovary (CHO) cells in the presence of DFMO. Coupled with our finding of no significant increase in apoptosis, these data indicate that polyamine depletion in IEC-6 cells caused cell cycle arrest.

Progression through the cell cycle is mediated by a phylogenetically conserved family of protein kinases known as CDKs. CDKs are composed of a catalytic subunit and a requisite positive regulatory subunit termed a cyclin (44). The CDK activities that govern cell cycle progression require coordination and regulation. In most cases, positive regulation is mediated at the level of cyclin accumulation (22, 25, 42). Negative regulation of CDK activity is achieved by phosphorylation of the catalytic subunit or via the binding of CDK inhibitory proteins that include p21^{Waf1/Cip1} and p27^{Kip1} (41). Increased levels of CDK inhibitory proteins bind to cyclin-CDK complexes and render these complexes inactive. CDK inhibitory proteins have been implicated in negative regulation of the cell cycle by both internal and external signals (19, 54). Treatment of IEC-6 cells with DFMO increased the levels of p27^{Kip1} and p21^{Waf1/Cip1} (Fig. 4, B and C). The increase in p21^{Waf1/Cip1} was significantly greater than the increase in p27^{Kip1}. Induction of p21 has been reported to be both p53 dependent and p53 independent based on the type of stimulus (34). Activation of p53 turns on the transcription of p21^{Waf1/Cip1}, which binds to and inhibits CDKs, causing the accumulation of the hypophosphorylated form of retinoblastoma protein. This form of retinoblastoma protein binds to E2F, which is a specific transcription factor for S phase gene expression, prevents the release of E2F, and blocks the G₁-S transition (12). Polyamine-depleted cells showed a significant induction of p53 protein (Fig. 4A), suggesting p53-dependent induction of p21^{Waf1/Cip1}.

The MAPK and SAPK pathways, which include the ERKs and JNK, respectively, link cell surface and nuclear events. Downstream effects of activated MAPK include phosphorylation of the substrates c-Fos and c-Jun, which in turn stimulates cell cycle progression. The current experiments clearly demonstrate that polyamine depletion decreased ERK-2 protein level while increasing ERK-2 activity. Addition of putrescine maintained the basal level of ERK-2 activity (Fig. 6). In contrast, ERK-1 protein was not affected by polyamine depletion, but ERK-1 activity also was increased (Fig. 5). This was a surprising and unanticipated correlation between increased MAPK activity and growth inhibition. These results are in contrast to the existing paradigm, which suggests concomitant increases in MAPK activity and cell proliferation. Pumiglia and Decker (45) first described a novel paradigm in which the MEK/MAPK pathway negatively regulates CDK activity and mediates cell cycle arrest. Soon thereafter, constitutively active MEK-1 was shown to induce epithelial differentiation and growth inhibition in Madin-Darby kidney cells (49) and MAPK was reported to mediate inhibition of proliferation in smooth muscle cells (3).

Because polyamine depletion inhibited cell growth and induced MAPK, we presumed that DFMO might cause stress and induce stress-regulated kinase (JNK) activity. DFMO treatment completely depleted putrescine within 3 h, whereas spermidine and spermine depletion was evident after 24 h and 3 days, respectively, in cultured intestinal epithelial cells (36). We observed a sustained increase in JNK activity when cells were grown in the presence of DFMO. Increased JNK activity was the earliest event observed (Fig. 8) and remained elevated up to 4 days (Fig. 7). Taken together, these results indicate a link between cell growth and activation of MAPK and SAPK. Recently, a role for oncogenic Ras and the MAPK pathway in p53 modulation and function has been revealed in both human and rodent cells. High expression of Ras or activation of the Mos/MAPK pathway induces wild-type p53 levels and causes a permanent growth arrest, similar to cellular senescence (16, 52). In a cell line defective in the MAPK pathway and in p53 expression, increased expression of ERK-2 restores the normal level of p53, clearly placing ERK-2 in a pathway that regulates the steady-state level of p53. MAPK has been shown to phosphorylate residue 73 or 83 of murine p53 in vitro (2, 38); p53 is involved in several different aspects of cell cycle arrest, apoptosis, control of genome integrity, and DNA repair (64, 65). Sustained increases in JNK also result in apoptosis in some cell lines by activating p53 and inducing CDK inhibitor proteins (8, 23, 32, 52, 64).

In summary, our results show that polyamine depletion of IEC-6 cells leads to cell cycle arrest in G₁ without causing apoptosis. Inhibition of growth was accompanied by increased levels of p21, p27, and p53. Taken together, these data suggest that in the absence of polyamines increased MAPK/JNK induces p53, which in turn may increase the transcription of p21, inhibiting CDK and blocking cell cycle progression. This is the first report that polyamine depletion activates signal transduction pathways leading to cell cycle arrest.

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